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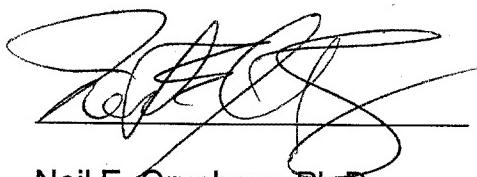
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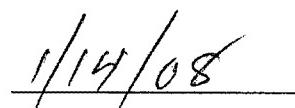
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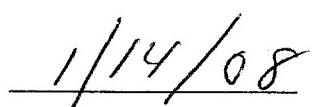
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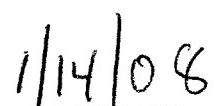
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ABSTRACT

Title of Thesis: Sex Differences in Nicotine Withdrawal in Adult Male and Female Rats

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Gender differences have been observed in difficulty quitting smoking. A rodent model of nicotine withdrawal has been used by several laboratories, but only in males. Nicotine withdrawal in male and female adult rats was examined in a dimly-lit, comfortable environment and a brightly-lit, uncomfortable environment. Ninety-six Sprague-Dawley male and female adult rats received 7 days continuous subcutaneous infusion via Alzet osmotic pumps filled with saline or 3.16 mg/kg of nicotine hydrogen tartrate. Behavioral observations were made before, during, and after nicotine or saline administration. Cessation of nicotine administration caused a significant increase in withdrawal behaviors in male and female rats in both environments. In the dimly-lit environment, female rats showed more withdrawal behavior than male rats, but there was no drug x sex interaction. In the brightly-lit environment, there was no male-female difference in withdrawal. Nicotine withdrawal was greater in the brightly-lit environment, and was more pronounced in female rats.

The Effects of Nicotine Withdrawal in Adult Male and Female Rats

by

Kristen R. Hamilton

Master's Thesis submitted to the Faculty of the
Department of Medical and Clinical Psychology
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INTRODUCTION

Cigarette smoking is the leading cause of preventable death in the United States, and leads to significant health consequences, including cardiovascular diseases, cancers, and respiratory diseases (CDC, 2006). The health risks associated with cigarette smoking are well-publicized in our society. Despite knowledge of these health consequences, one out of every five adults in the U.S. smokes cigarettes (CDC, 2006). Rates of cigarette smoking are slightly higher among men (23.9%) than among women (18.1%).

Fortunately, health risks associated with cigarette smoking decrease after smoking cessation (USDHHS, 1990). However, whereas men and women smoke at somewhat similar rates, women are less successful than men at quitting smoking (Perkins, 2001; Swan, Jack, & Ward, 1997; Wetter, Kenford, Smith, Fiore, Jorenby, & Baker, 1999; CDC, 2001). In population-based data, the quit ratio (former smokers to ever smokers) is consistently higher for men (52%) than for women (47%) (CDC, 1994). This ratio is consistent with research on smokers seeking treatment, in which men had higher cessation rates than did women (Fiore et al., 1994; Wetter et al., 1994). People continue to smoke cigarettes largely because of nicotine, a highly addictive drug that plays a major role in reinforcing the maintenance of tobacco use (USDHHS, 1988, Grunberg, Faraday, & Rahman, 2001; Grenhoff & Svensson, 1989). People smoke cigarettes to regulate the level of nicotine in their bloodstream (Creighton, 1976; Wade, 1972). Gender differences in smoking cessation could result from differences in level of physiological addiction to nicotine, which may be reflected

in severity of withdrawal symptomatology. The human literature is mixed, with some research reporting no gender differences in withdrawal symptomatology (e.g., Svikis, Hatsumaki, Hughes, Carroll, & Pickens, 1986), and other research reporting more nicotine withdrawal symptoms in women (e.g., Shiffman et al., 1979). An animal model of nicotine withdrawal provides a means of conducting a true experiment and ruling out effects of self-report and expectation that are inherent in human studies. A rodent model of nicotine withdrawal is used in the present experimental series to determine whether withdrawal-related behaviors (as an index of nicotine addiction) differs between males and females. Additional factors that have been found to predict smoking cessation success in humans include the presence of rules against smoking in the home, likelihood of having switched to light cigarettes for health concerns, older age, being married or living with a partner, being of non-Hispanic White ethnicity, and having at least a college education (Lee and Kahende, 2007). The factors affecting nicotine withdrawal that translate most readily to an animal model, age and ethnic (genetic) differences, have been examined experimentally (e.g., Perry, Hamilton, Shafer, and Grunberg, in preparation).

The present experimental series is a comparison of nicotine withdrawal in males and females in a rodent model. As background for this work, the introduction summarizes health consequences of cigarette smoking, benefits of cessation, gender differences in smoking cessation success, possible reasons for gender differences in smoking cessation, and limitations of available research

regarding gender differences in smoking cessation. Next, each of two related animal experiments designed to address questions raised in the introduction are presented with hypotheses, methods, results, and discussion. Then, in a general discussion, the implications and limitations are addressed.

Health Consequences of Cigarette Smoking

Cigarette smoking causes many adverse health consequences, including cardiovascular diseases (CVD), cancers, respiratory diseases, and immunosuppression. With regard to cardiovascular diseases, smoking increases the risk of stroke and Coronary Heart Disease (CHD) by 100%, increases the risk of death from undiagnosed CHD by 300%, increases the risk of death from peripheral artery disease by over 300%, and increases the risk of aortic aneurysm by 400% (CDC and World Health Organization, 2004). In 2000, it was estimated that 4.83 million premature deaths worldwide were attributable to smoking (Ezzati & Lopez, 2003). Of cancers, 90% of lung cancer in men and 80% of lung cancer in women is attributable to cigarette smoking, and smoking increases the risk of cancer of the oral cavity, pharynx, larynx, esophagus, and bladder (USHDDS, 2004). It is estimated that of the 4.83 million deaths that occurred in 2000 in the world, 1.69 million deaths were caused by cardiovascular disease, 0.97 million deaths were caused by chronic obstructive pulmonary disease (COPD), and 0.85 million deaths were caused by lung cancer (Ezzati & Lopez, 2003). In 2000, 18.1% of U.S. deaths (435,000) were attributable to smoking (Mokdad, Marks, Stroup, & Gerberding, 2004).

Health Benefits of Smoking Cessation

Fortunately, the health risks associated with cigarette smoking decrease after smoking cessation (USDHHS, 1990). The risk of lung cancer is reduced by 30 to 50% ten years after smoking cessation. The risk of CHD is halved one year after cessation and equals that of never smokers 15 years after cessation. The risk of stroke is equal to that of people who have never smoked 5 to 15 years after smoking cessation, and the risk of COPD is equal to that of never smokers after sustained abstinence from cigarette smoking. Women who stop smoking before pregnancy or during the first 3 to 4 months of pregnancy reduce their risk of having a low-birth-weight infant to that of women who never smoked. In addition, smoking cessation decreases the risk for all other cancers, heart attack, and chronic lung disease. In summary, smoking cessation is immensely beneficial and reduces the risk of diseases associated with cigarette smoking (USDHHS, 1990). Unfortunately, women are less successful at smoking cessation than men (Perkins, 2001; Swan, *et al.*, 1997; Wetter, *et al.*, 1999; CDC, 2001). Comparing male and female withdrawal symptomatology in a rodent model of nicotine abstinence syndrome will help determine whether gender differences in smoking cessation success result from sex differences in physiological addiction to nicotine. This information may help to inform treatment strategies for smoking cessation in women, thereby improving their chances of successful smoking cessation.

Smoking Health Consequences Unique to Women

Whereas both men and women smokers suffer from adverse health consequences, female smokers may have an even higher risk than male smokers for lung diseases and CVD (Devessa, Bray, Vizcaino, & Parkin, 2005; Risch, Howe, & Jain, 1993; Neugut & Jacobsen, 2006; CDC, 2001). Additionally, there are health risks associated with cigarette smoking that are unique to women, such as breast cancer, menstrual irregularities, and fertility problems. Cigarette smoking during pregnancy is associated with a variety of health risks to the fetus, including increased risk of spontaneous abortion, Sudden Infant Death Syndrome (SIDS), and low birth weight (Perkins, 2001). Therefore, smoking cessation has additional health benefits for women. However, although women are at increased risk for disease states associated with cigarette smoking, women are less successful at smoking cessation (Perkins, 2001). As women have unique smoking health consequences, but at the same time are less successful at quitting smoking, the present research is especially valuable to determine the cause of the lower success rate of females in smoking cessation, which will inform treatment strategies for women.

Gender differences in smoking cessation

Gender differences in smoking cessation success could result from a variety of factors, including psychological, social, environmental, and biological factors. For example, women experience depression at roughly twice the rate of men (Robins & Rieger, 1990), and addiction to substances such as nicotine frequently co-occurs with depression (Volkow, 2004; DSM-IV-TR, 2000). It is

also possible that environmental variables may differentially affect smoking behavior in males and females. Differences in biological effects, a primary focus of the present research, center on addiction to nicotine. Because withdrawal severity in rodents is unlikely to be affected by psychosocial factors that may affect smoking relapse in humans (e.g. concerns about weight gain), any sex differences in withdrawal symptomatology in rats are likely to reflect a biological cause. Therefore, if there are sex differences in nicotine withdrawal in rats, then there is likely to be a biological cause for the gender difference in human cessation success. Likewise, an absence of sex differences in the rodent model will suggest that the human gender difference in cessation success does not result from biological factors, but instead results from personal human factors that are not present in rats. In addition, it is important to determine if there are differences in nicotine withdrawal symptoms in different environments and whether there are environment x sex interactions in nicotine withdrawal. The information gained from these experiments will inform treatment cessation strategies for men and women.

Nicotine

Nicotine is the addictive psychoactive chemical found in all tobacco products that is largely responsible for smoking maintenance. Nicotine, an alkaloid from the nightshade family, makes up 0.3 to 5% of the tobacco plant. Nicotine acts as a stimulant in mammals, although some smokers also report relaxing effect of nicotine (Kozlowski, Henningfield, & Brigham, 2001). Nicotine acts on nicotinic cholinergic receptors in the Central and Peripheral Nervous

Systems, and stimulates release of the neurotransmitters dopamine, gamma-aminobutyric acid (GABA), norepinephrine, epinephrine, and beta-endorphin in the brain (Barik & Wonnacott, 2006; Koob & LeMoal, 2006). Marked nicotine withdrawal in humans lasts for approximately ten days, and symptoms include tension, irritability, headaches, and increased appetite and weight gain (Shiffman et al., 2006). Craving for nicotine and weight gain may last for a year after cessation (Grunberg, 1985, 1987). Nicotine withdrawal symptoms are aversive and difficult to endure, and when people are trying to quit smoking, they often relapse in an attempt to escape from withdrawal symptoms. A greater intensity of withdrawal symptoms in females would make them even more likely than males to relapse. Finding such a sex difference would suggest a biological basis for the gender discrepancy in smoking cessation success.

Gender differences in nicotine withdrawal symptomatology

Patten and Martin (1996) reviewed 15 prospective studies that attempted to determine whether the severity of nicotine withdrawal was predictive of smoking cessation and relapse (e.g. Covey, Glassman & Stetner, 1990; Gritz, Carr, & Marcus, 1991; Gunn, 1986). Patten and Martin concluded that overall, the results of the studies they reviewed were equivocal, because 6 of the 15 studies showed a relationship between withdrawal and smoking cessation success outcome. Interestingly, responses to stress may affect likelihood of smoking relapse differently in men and women, with stress increasing the severity of nicotine withdrawal symptoms in women. Al' Absi (2006) reported that intensity of withdrawal symptoms after exposure to acute stress was a consistent

predictor of smoking relapse in women, whereas attenuated cortisol and adrenocorticotropic hormone (ACTH) responses to stress are predictive of smoking relapse in men. Because nicotine withdrawal symptom severity may play a role in smoking relapse in smokers attempting to quit, any sex differences in nicotine withdrawal symptom severity could contribute to the gender difference in smoking cessation success, in which women have worse smoking cessation outcomes than men.

The human literature on gender differences in nicotine withdrawal symptoms is mixed, with some researchers finding no gender differences in self-reported withdrawal symptomatology (e.g., Svikis, et al., 1986; Hughes, 1992), and other researchers finding more self-reported nicotine withdrawal symptoms in women than men (e.g., Shiffman, 1979). It is noteworthy that there are no reports of greater withdrawal symptoms in men than women. Hughes, Higgins, and Hatsumaki (1990) observed that the studies in which women self-report greater withdrawal severity than men are retrospective. Pomerlau, Tate, Lumley, and Pomerlau (1994) conducted a retrospective and prospective study of self-reported nicotine withdrawal symptoms in women and men. They found that women reported more withdrawal than men retrospectively, but both sexes reported the same level of withdrawal symptom severity prospectively (while in withdrawal). While the Pomerlau (1994) prospective study suggests similar withdrawal symptomatology in men and women, a true experiment using an animal model is needed in order to compare nicotine withdrawal in males and females.

Examining nicotine withdrawal with a rodent model allows for the random assignment of animals to nicotine groups, a condition that is necessary for a true experiment to determine whether nicotine withdrawal symptoms differ between males and females. In addition, use of an animal model limits effects such as self-report, expectation, and recall bias. For these reasons, a rodent model of nicotine withdrawal was used in the present experiment to determine if there are biologically-based sex differences in withdrawal.

Gender differences in effects of the environment

Gender differences exist in stages of cigarette smoking other than withdrawal, such as maintenance. Gender differences have been reported in amount of smoking, with men smoking more cigarettes than women (Grunberg, et al., 1991; Perkins, Donny, & Caggiula, 1999; Halfon, Kark, Baras, Friedlander, & Eisenberg, 1982). In a study of nicotine nasal-spray self-administration in smokers trying to quit, men randomly assigned to nasal spray self-administered it at twice the rate of those assigned to placebo spray, whereas there was no difference in self-administration behavior between women who were randomly assigned to placebo and nicotine (Perkins, Grobe, D'Amico, Fonte, Wilson, & Stiller, 1996). These results suggest that women who are quitting smoking may find nicotine *per se* less reinforcing than do men, but instead may be reinforced by smoking-related stimuli and environmental context. Women self-report that they find smoking-related stimuli, such as 'hand-mouth activity' more rewarding than men (Parrott & Craig, 1995). Research from Perkins (1999) suggests that environmental context plays a greater role in determining the perception of

nicotine effects in women than men. Because environmental context is important for the perception of nicotine effects in women, it may play an important role in nicotine withdrawal in women as well.

Al' Absi (2006) reported that intensity of withdrawal symptoms is a consistent predictor of smoking relapse in women, whereas hormonal associations are consistent predictors of smoking relapse for men. As women are more likely to relapse as a result of withdrawal symptom intensity, a greater intensity of withdrawal symptoms in females would make females even more likely than males to relapse. Therefore, if females experience nicotine withdrawal in an environment that exacerbates their withdrawal symptomatology (e.g., a stressful environment), they would be at a greater risk of relapsing. By contrast, in males, a blunted hormonal response to stressors was associated with nicotine withdrawal, suggesting that the mechanism by which stress increases risk for smoking relapse (e.g. Wills, Sandy, & Yeager, 2002) differs in men and women (al' Absi, et al. 2006).

Effects of Circulating Sex Hormones

In animal studies, estrogen enhances drug-seeking behavior in many phases of drug abuse that show sex differences, but there is little evidence for a role of other sex hormones, such as progesterone and testosterone, in drug-seeking behavior (Carroll et al., 2004). For example, estrogen facilitates self-administration of cocaine and heroin, thereby enhancing the acquisition phase of cocaine and heroin addiction (Lynch, et al., 2002). Donny et al. (2000) reported that estrous cycle phase had no effect on nicotine self-administration. However,

the authors speculated that heterogeneity within each cycle phase may have limited their ability to detect a relation between discrete stages and self-administration (Donny, et al. 2000). Heterogeneity within each cycle phase can result from variance that occurs when female rats are allowed to cycle naturally, rather than through pharmacological synchronization so that all rats experience cycle phases at the same time. The variance that results from allowing females to cycle naturally decreases an investigator's power to detect an effect of estrous cycle on behavior. For example, Roberts et al. (1998) reported an effect of estrous cycle phase on ethanol self-administration in rats when cycles were synchronized pharmacologically, with lower ethanol intake occurring during the estrous phase, although they found no effect of estrous cycle phase in rats with free running estrous cycles. Because estrous cycle phase affects seeking and administration of other drugs, it is possible that estrous cycle phase affects severity of nicotine withdrawal in female rats. To examine the effect of circulating sex hormones on nicotine withdrawal in females, estrous cycle phase was assessed daily in the present experiment.

Rodent Model of Nicotine Withdrawal

The rodent model of nicotine withdrawal used in the present experimental series was developed by the Malin group (1992). Previous work from the Malin group was focused on morphine dependence, in which they discovered that some of the most widespread and useful types of models of morphine dependence were those in which rats spontaneously exhibited quantifiable unusual behaviors during abstinence (Gianutsos, Drawbaugh, & Lal, 1975; Malin

et al., 1988). With the aim of developing a laboratory model of nicotine withdrawal, the Malin group conducted extensive pilot studies in which they took various physiological measurements and recorded all countable behavioral events before, during, and after nicotine infusion (Malin, 2001). They identified certain behaviors, termed “somatic behavioral signs,” as being selectively elevated during the withdrawal phase, particularly whole body shakes, abnormal grooming, abnormal posture, ptosis (slackening of the jaw), mouthing/teeth chattering, eyeblinks, and diarrhea.

Several lines of evidence support the validity of the present model as a representation of nicotine withdrawal syndrome (Kenny & Markou, 2001). First, when nicotine is chronically administered and then withdrawn from rats, they display more somatic behavioral signs than when these same subjects were nicotine naive, immediately prior to the termination of nicotine administration, after the recovery from withdrawal or compared to saline-treated control rats (Malin *et al.*, 1992). Second, the severity of the somatic behavioral signs was proportional to the amount of nicotine to which the animal was exposed, with animals receiving higher concentrations of nicotine displaying more behavioral signs. Third, nicotine administration reverses withdrawal behavioral signs in rats undergoing nicotine withdrawal, which demonstrates that tonic activation of nicotinic cholinergic receptors (nAChR), which are upregulated when addiction develops, is critical to prevent the somatic behavioral symptoms (Malin *et al.*, 1992).

Although the rodent model is internally valid, issues of external validity, or "face validity," remain (Malin, 2001). For one, the rodents are exposed to a high rate of nicotine (3.16 mg/kg/day) to compensate for the fact that the period of nicotine exposure is only one week long. However, blood nicotine concentration in rats given 3.16 mg/kg/day nicotine is virtually identical to the concentration steadily maintained during waking hours by human subjects who smoked 30 high-yield cigarettes per day (Benowitz et al., 1982).

A second difference that threatens external validity is that nicotine is delivered continuously in the laboratory model, while humans self-administer nicotine intermittently with cessation during sleeping hours (Malin, 2001). Additional differences are the route of drug administration and the presence of nonnicotine ingredients in tobacco smoke (Malin, 2001). In light of the many differences between nicotine administration in humans and animals, phenomena discovered in animal models should be tested empirically in humans before application to a clinical setting.

Malin's rodent model of nicotine withdrawal has proven to be reliable because it has produced consistent results across a number of experiments of nicotine withdrawal from both the Malin group (1993, 1994, 1996, 1998, 2001) and other laboratories (Caroni et al., 2000; Epping-Jordan et al., 1998; Hildebrand et al., 1997, 1998; Watkins et al., 2000).

Present Experiment

A rodent model of nicotine withdrawal was used in the present experimental series to determine whether level of physiological addiction to nicotine differs between males and females. Malin et al. (1992) developed an animal model to examine nicotine withdrawal in rats. Several laboratories, including ours, have used this model and all have reported that male rats display distinctive withdrawal behaviors after receiving and then discontinuing nicotine administration (Malin et al., 1992; O'Dell et al., 2004, Phillips, et al., 2004). No published studies, however, have used this model to examine nicotine withdrawal in female rats.

The purpose of the present series of experiments was to examine sex differences in nicotine withdrawal in an animal model in two different environments. Because women report greater difficulty with smoking cessation, it was hypothesized that adult female rats would display more withdrawal behaviors than males, suggesting more severe nicotine withdrawal and a higher level of addiction. Specifically, it was hypothesized that females would have a greater magnitude of withdrawal behavior than males, and that females would have a greater duration of withdrawal behavior than males. It is also possible that males and females would have no differences in the magnitude and duration of withdrawal behaviors, which would suggest that the gender difference in human smoking cessation success results from factors other than biological ones, such as social or personal factors.

Research from Perkins (1999) suggests that environmental context is more important for the perception of nicotine effects in women than men. As the environment may have a greater effect in women on the perception of nicotine's effects, it may play a more important role in nicotine withdrawal in women than in men as well. As Perkins' (1999) research emphasizes the importance of the environmental context, the environment in which withdrawal behavior was observed was given special consideration in the present experimental series. Although it is not stated in any published reports (Malin, 1992, 1994, 2006), Malin observed rats in cages without bedding in a brightly lit room (Dr. Malin, personal communication, 2006). This environment differed from the environment used in Experiment 1, in which rats were observed in cages with bedding in a dimly-lit room. The bright lights in the room may have been a source of environmental stress for the rats. Because rats are nocturnal animals that naturally avoid the light, exposure to bright light is a stressor for rats, and has been used as a stressor in experimental investigations (e.g., Slawecki, 2005). As stress results in increased nicotine administration, it is possible that environmental conditions can alter nicotine withdrawal. For these reasons, nicotine withdrawal was observed in two different environments in the present experiment. Results are valuable to elucidate possible reasons for the gender difference in smoking cessation success, and may help to improve cessation treatment strategies.

The present research project included two experiments to compare nicotine withdrawal in male and female rats in different environments. In Experiment 1, withdrawal observations were conducted in a dimly-lit room in

cages with bedding to model the home environment as closely as possible, and to be consistent with other experiments conducted in our laboratory (Phillips, Schechter, & Grunberg, 2004). In Experiment 2, withdrawal observations were conducted in a brightly-lit room in cages without bedding, to be consistent with similar experiments conducted by other laboratories (e.g. Malin, 1992, 1994, 2006; Odell, 2004, 2006). Examining nicotine withdrawal in two different environments allowed for comparison of reported experiments in the research literature and addressed the question of whether environmental differences altered effects of nicotine withdrawal in this paradigm.

Hypotheses:

Drug condition

Hypothesis 1. It was hypothesized that rats that received nicotine would display more withdrawal than rats that received saline after pump removal.

Rationale: Previous research (e.g. Malin, et al. 1992, Koob et al., 2004, O'Dell et al., 2004) reported a significant main effect of nicotine administration on withdrawal behavior in adult male rats. While similar research has not been conducted in female rats, human studies indicate that females report withdrawal symptoms after the cessation of chronic nicotine use (e.g. Perkins et al., 2006).

Sex

Hypothesis 2. It was hypothesized that female rats would display more withdrawal behaviors overall than males.

Hypothesis 2a: It was hypothesized that female rats would have a greater magnitude of withdrawal behavioral symptoms than male rats.

Hypothesis 2b: It was hypothesized that female rats would have a greater duration of withdrawal behavioral symptoms than male rats.

Rationale: While a study of sex differences in nicotine withdrawal behaviors has not been conducted previously, marked sex differences have been demonstrated in rats in many other phases of drug addiction. There is a faster acquisition of nicotine self-administration at a lower nicotine dose in females (e.g. Donny et al., 2000). Female rats exceed males in escalation, dysregulation, binge-like patterns of cocaine administration (Carroll, Lynch, Roth, Morgan, Cosgrove, 2004), and reinstatement and relapse (e.g., Carroll & Comer, 1996). Donny et al. (2000) report that on several measures that may reflect motivation to obtain a drug, females showed a higher motivation to obtain nicotine than males. Sex differences also have been reported in experiments with other drugs. For example, recent research reports that females had greater locomotor activity responses to administration of the stimulants amphetamine and methamphetamine (Milesi-Hallé, McMillan, Laurenzana, Byrnes-Blake, & Owens, 2007). As there are sex differences in other phases of drug abuse, with females having greater self-administration, dysregulation, reinstatement, and relapse, it was hypothesized that females would have more withdrawal behaviors than males after cessation of continuous nicotine administration.

Body weight

Hypothesis 4. It was hypothesized that nicotine administration would decrease body weight gain in males and females and nicotine cessation would result in a greater rate of weight gain. In addition, these effects would be greater in female than male rats.

Rationale: Previous nicotine research has demonstrated the inverse relationship between nicotine and body weight, with nicotine exhibiting a greater effect upon body weight in females than males (Grunberg, 1982; Grunberg, 1992; Winders & Grunberg, 1989). These effects are dependent upon dosage, with a high, 12 mg dose of nicotine decreasing body weight in females below pre-drug values (Grunberg, 1986).

Experiment 1

Male and Female Rat Nicotine Withdrawal Observed in a Dimly-Lit Environment in Cages with Bedding

Overview

The purpose of this experiment was to examine the effect of nicotine withdrawal in male and female adult rats in a dimly-lit environment in cages with bedding. The withdrawal behaviors identified by Malin et al. (1992) were used to examine nicotine abstinence syndrome in the rats 20 hours after removal of subcutaneous (SC) nicotine pumps that had been inserted one week prior. In the present experiment, the observation room was dark to be similar to the home-cage environment and to minimize stress from the environment, following the procedures used by Phillips et al. (2004) to study nicotine withdrawal in male rats. The experiment was divided into three phases: before, during, and after nicotine or saline administration. Rats were observed in standard shoebox cages (42 x 20.5 x 20 cm) with cage bedding four times in a dark room with low lighting provided by a lamp for 15 minutes each: once during baseline phase, once during drug administration, and twice during the withdrawal phase. The independent variables in the present experiment were sex (male, female) and drug condition (nicotine, saline), and the dependent variables were observed withdrawal behaviors, body weight, and locomotor activity. The present experiment was a 2 (sex) x 2 (drug condition) x 4 (time) mixed model, with sex and drug condition as between-subjects factors and time of observation as the within-subjects factor.

The days on which each measure occurred are illustrated in the following timeline:

Day 1: rats arrive
Days 2-3: gentling
Day 4: estrous measurements begin (females)
Day 6: baseline locomotor activity
Day 7: baseline withdrawal observations
Day 8: pump implant
Day 14: nicotine withdrawal observations
Day 15: pump explant
Day 16: withdrawal day one observations
Day 17: withdrawal day two observations
Day 24: estrous measurements end (females), euthanasia

Subjects

Subjects were 24 female and 24 male Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA). The female and male rats were run in two cohorts. Animals were individually housed in standard polycarbonate shoebox cages (42 x 20.5 x 20 cm) on hardwood chip bedding (Pine-Dri). Animals had continuous access to rodent chow (Harlan Teklad 4% Mouse/Rat Diet 7001) and water during all phases of the study in the home cages. Housing rooms were maintained at 23 degrees Celsius at 50% humidity on a 12 hour light/dark cycle (lights on at 0800 hours). A reverse light cycle was used so that the rats, which are nocturnal animals, would be tested during their active phase. Upon arrival, the rats were approximately 67 days old, an age in rats that is analogous to adulthood in humans. At the beginning of the experiment, the females weighed an average of 186.5 grams and the males weighed an average of 303.7 grams. Using rats that were approximately 67 days old ensured that all

rats were adults, because rodent adulthood begins at approximately 40 to 50 days (Douglas, Varlinskaya, & Spear, 2004).

METHODS

Baseline Phase

The baseline phase lasted for one week (7 days) after rats' arrival. In the first two days after arrival, rats were gentled by being held and petted for two minutes each so that they would be accustomed to being handled by humans, and acclimated to the activity chambers. Daily collection of estrous samples commenced on the third day of the baseline phase. Rats were weighed using a standard laboratory scale every other day, with the first day of body weight measurement on the first day. The rats were weighed every other day for the duration of the experiment. On the sixth day rats were placed in the individual activity chambers for one hour to record horizontal and vertical activity and center time. Baseline observations were conducted on the seventh day of baseline.

Observation Period

Observations were conducted on the last day of baseline phase, the last day of nicotine phase, and the first two days of the withdrawal phase (20 hours and 44 hours after pump explant). During the observation period, raters recorded occurrences of nicotine withdrawal behaviors (Malin, et al., 1992, 1994, 2006; Phillips et al., 2004; O'Dell et al., 2004, 2006) which include whole body shakes, abnormal grooming, abnormal posture, ptosis (slackening of the jaw), and diarrhea. The observations were conducted in a dark room illuminated by one 60 watt light bulb, and each observation period lasted for 15 minutes. To train the raters, all withdrawal behaviors were initially explained to the raters, and then the raters observed the rats alongside experienced raters. By discussing

the observed behaviors with the experienced raters between trials, the trainees learned to identify all withdrawal behaviors. When raters reached an inter-rater reliability level of approximately 90% with the experienced raters, they were allowed to rate independently. Throughout the experiment, raters continued to discuss behaviors at the end of each observation session to enhance inter-rater reliability.

Animals were observed in clean standard polycarbonate shoebox cages (42 x 20.5 x 20 cm) on hardwood chip bedding (Pine-Dri) in a separate room from the housing room. Occurrences of withdrawal behaviors were counted. These behaviors included abnormal posture, abnormal grooming, whole body shakes, ptosis, mouthing/teeth chattering, and diarrhea. Other behaviors that appeared abnormal were counted as "other." A detailed description of behaviors, which was distributed to raters on each observation day, is included in Appendix A.

Locomotor activity measurement

Rats' locomotor activity was measured to account for differences in overall general activity. Measurement of locomotor activity revealed whether nicotine rats differ from saline rats in overall general locomotor activity. If rats do not differ in overall locomotor activity, then differences in the occurrence of specific behavioral signs of withdrawal must result from cessation of nicotine administration, and is not a reflection of increased overall activity in one group of rats.

Estrous measurement

Estrous measurements were conducted on the female rats to determine if withdrawal behavior was related to phase of the estrous cycle. In the estrous cycle, which is analogous to the human female's menstrual cycle, the epithelial cells lining the walls of the vagina change shape and size in correspondence with four phases: proestrous, estrous, metestrous, and diestrous. One estrous cycle is complete in four to six days, and investigators can determine what phase of the estrous cycle a rat is in by examining the vaginal epithelial cells (Marcondes, Bianchi, & Tanno, 2002).

Estrous measurements were taken daily at 0900 hours for 21 days, beginning 3 days after the rats' arrival. A flushing technique was used to collect vaginal epithelial cells from each rat. The flushing technique involved flushing rats' vaginal cavities with saline to collect epithelial cells. Rats were held in a secure position with the vagina exposed. A bulb was placed on the large end of a flamed pipette and saline was withdrawn from a container. The pipette was used to expel saline into and withdraw saline from the cavity approximately three times. The pipette tip containing approximately three ml of saline was inserted into the vaginal cavity, avoiding contact with the walls.

When the saline was withdrawn from the vagina, it was filled with the epithelial cells that line the vaginal walls. The cells were placed on designated sections of microscope slides that were later dyed and rated by independent

raters to determine the phase of estrous cycle each rat was in at the time of measurement.

Estrous staining procedure

Estrous slides were dipped successively into methanol (7 minutes), water three times (3 minutes each), hematoxylin (4 to 5 minutes), alcohol (1 to 2 minutes), eosin (2 to 5 minutes), water (briefly), alcohol three times (1 to 2 minutes each), and lastly, xylene (1 minute). Cover slips were fixed to the slides using Permount, and the slides were allowed to dry. After drying, slides were viewed by independent raters under Reichert-Jung (Series 150) microscopes at 40 X magnification.

Drug Administration Phase

The drug administration phase began after the pump implant. During this phase, which lasted one week, rats received a continuous flow of nicotine bitartrate or physiological saline (0.9% NaCl) at 5.25 microliters/hour for a dosage of 3.16 mg/kg/day. Estrous measurements continued to be taken daily at 0900 hours. Nicotine observations took place on the sixth day of the drug administration phase in the manner described above. The drug administration phase ended with the pump explant, which took place from 1600 hours to 2000 hours on the last day of the nicotine phase, one week after pump implant.

Drug Administration and Surgery Procedure

From 1200 to 1400 hours on the 8th day after their arrival, rats were subcutaneously implanted with Alzet osmotic pumps (Model 2ML2) filled with nicotine bitartrate solution or 0.9% NaCl (physiological saline). The pumps

delivered a continuous flow of nicotine bitartrate at approximately 5 microliters/hour for a dosage of 3.16 mg/kg/day (expressed as nicotine base), because this dosage has been effective in producing a sufficient level of addiction after one week of continuous administration in other studies of nicotine withdrawal (e.g., Phillips et al., 2004).

Subjects were anesthetized individually in a plastic chamber with a continuous flow of oxygen (flow rate: 0.5 to 1.0 liters/minute) and 2 to 4% isofluorane gas into the chamber to induce anesthesia. When the animal showed lack of response to a tail-pinch test, the animal was removed from the chamber and a 3 x 5 cm area between the animals' withers was shaved and cleaned with an iodine-based antiseptic (Betadine). The rats' anesthesia-induced unconscious state was maintained via a nose cone and tube that delivered a combination of 0.25 to 3% isofluorane and oxygen from the induction chamber. A 2 cm horizontal incision was made with blunt-nosed surgical scissors, a subcutaneous pocket was created by spreading the subcutaneous tissues with the scissor tips, and a mini-pump was inserted with the flow modulator oriented towards each subject's tail. The incision was closed with 9 mm stainless steel wound clips. The entire surgical procedure took approximately 4 minutes.

Observations

Observations during the drug administration phase were conducted in an identical manner to observations during the baseline phase.

Withdrawal Phase

Withdrawal phase began immediately after pump explant. Rats were observed 20 hours after pump removal, in the middle of the optimal 18 to 22 hour window for observing withdrawal behaviors described by Malin et al., (1992), Phillips et al (2004), and O'Dell et al. (2004, 2006). The locomotor activity parameters of horizontal and vertical activity and center time were collected for one hour in the locomotor chambers on Withdrawal Day One, following observations. The second and third withdrawal observations took place 24 and 48 hours after the first withdrawal observation. Estrous and body weight measurements continued to be carried out nine days after the first withdrawal day.

Surgery Procedure

The pumps were explanted after 7 days of nicotine or saline administration. Before explanting the pumps, anesthesia was induced using the same procedure as described above. A 2.5 x 4 cm area surrounding the implanted minipump was shaved and cleaned with Betadine. A 1.5 cm incision was made at the base of the implanted minipump and the minipump was removed. The incision was closed with 9mm stainless steel wound clips. The entire surgical procedure took approximately 3 minutes.

Observations

Observations during the withdrawal phase were conducted in an identical manner to observations during the baseline phase.

Locomotor activity

Measurements of locomotor activity were collected during the withdrawal phase in an identical manner to locomotor activity measurement collection during the baseline phase.

Materials and Equipment

Activity Chambers

Animals were placed in individual electronic physical activity monitoring chambers of the Omnitech Electronics Digiscan infrared photocell system [Test box model RXYZCM (16 TAO); Omnitech Electronics, Columbus, OH] for one hour to measure open field locomotor activity and record vertical and horizontal movement via a grid of infrared light beams. Equally spaced beams traversed the plastic arenas (40 x 40 x 30 cm) from front to back and left to right. Fifteen pairs of infrared photocells are located every 2.5 cm from left to right and from front to back in a plane 2 cm above the floor of the chamber to measure horizontal movement. An additional 15 pairs of infrared photocells are located every 2.5 cm from left to right 10.5 cm above the floor of the chamber to measure vertical movement. Dependent variables included center time, horizontal activity, and vertical activity. The body of the animal placed in the chambers broke the beams, revealing any horizontal and vertical movement and center time. These parameters were automatically calculated based on beam breaks in twelve five minute bins, and automatically transferred to a personal computer via an Omnitech analyzer (Model DCM-8-BBU). Analyses were performed on total

scores for each dependent variable which were calculated by summing the scores for each parameter recorded every five minutes. The apparatus monitored animal activity continuously for a total testing period of 1 hour, collecting data in 5 minute bins. Several activity-related variables were examined including total horizontal activity, total vertical activity, and center time.

Estrous measurements

The materials for the estrous measurements included a slide tray, flamed disposable 5 ¾" Fisher brand pasteur pipets, 2 ml Sigma-Aldrich brand rubber bulbs, Fisher brand frosted microscope slides divided into eight sections each with a diamond-tipped pen, and 0.9% NaCl (physiological saline).

Estrous slide staining

The materials used for estrous slide staining included the chemicals 100% Methanol, Hematoxylin, Eosin Y, Permount, Xylene, and 100% Alcohol (200 proof). The non-chemical materials used included disposable pipettes, rubber bulbs, blunt forceps, a slide tray, a funnel, and Coplin jars.

RESULTS

Data analytic strategy

Withdrawal observations. Withdrawal behavior data were analyzed with Univariate Analyses of Variance (ANOVAs) at baseline (BL) and during saline or nicotine administration. There were no differences between drug conditions at BL, but there was a sex difference in total withdrawal behaviors at baseline [$F(1,44)=7.438$, $p<0.01$]. There were no main effects of drug condition or sex at the nicotine observations. Therefore, withdrawal behavior data on cessation days were analyzed using BL withdrawal behaviors as a covariate. Data were analyzed using Repeated-Measures Analysis of Covariance (ANCOVA) with BL as the covariate to control for differences existing in rat withdrawal behavior prior to the administration of drug or saline. Additionally, ANCOVAs using baseline withdrawal behaviors as a covariate were conducted for each of the two withdrawal days to determine how significant effects may have differed across withdrawal days. The withdrawal behavior results of Experiment 1 are displayed in Figure 1. For all analyses, time was the within-subject factor, and drug condition and sex were the between-subjects factors. All tests were two-tailed. Alpha levels were set at 0.05.

Body weight data. Body weight data were collapsed across the three phases (baseline, drug administration, and withdrawal) and the averages were analyzed with a repeated-measures ANOVA, using drug condition as the between-subjects factor and phase as the within-subject factor.

Estrous. Estrous data were analyzed using a Mixed Model approach (Arnold, 1992). The phases of the estrous cycle were effects and dummy coded and the data were analyzed to determine whether amount of withdrawal behavior displayed was related to estrous cycle phase.

Results

Withdrawal Observations

A repeated-measures ANCOVA, using baseline as the covariate, was used to compare withdrawal behaviors in male and female rats that received nicotine and saline across all observations (i.e., after two drug days). The analysis revealed a main effect of drug condition [$F(1,44)=16.39$, $p<0.01$], with rats that received nicotine displaying more withdrawal behaviors than rats that received saline, and a main effect of sex [$F(1, 44)=29.73$, $p<0.01$], with females displaying more withdrawal behavior than males. There was no sex x drug condition interaction in Experiment 1. The results of Experiment 1 are displayed in Figure 1.

Additionally, ANCOVAs using baseline withdrawal behaviors as the covariate were conducted for each of the two withdrawal days. The statistical analyses for all experiments are displayed in tables in Appendix A. There was no difference in baseline withdrawal behaviors between rats that later received saline and rats that later received nicotine, although there was a difference in baseline withdrawal behaviors between males and females [$F(1,44)=7.438$, $p<0.01$], with males displaying more withdrawal behaviors than females.

However, this difference at baseline was controlled for in all subsequent analyses using an ANCOVA with baseline withdrawal behaviors as the covariate. During drug (nicotine or saline) administration observations, there were no differences in withdrawal behaviors between male and female rats, nor were there differences between rats that had received nicotine or saline. Additionally, there was no sex x drug interaction in withdrawal behaviors at any of the observation time points.

Withdrawal Day One. An ANCOVA using baseline as covariate revealed a significant main effect for drug condition [$F(1, 44)= 15.31, p<0.01$], with the rats that had received nicotine displaying significantly more withdrawal behaviors than the rats that had received saline, and a main effect for sex [$F(1, 44)=29.33, p<0.01$], with females in both drug conditions displaying significantly more withdrawal behaviors than males in the corresponding conditions. There was no sex x drug condition interaction.

Withdrawal Day Two. An ANCOVA, using baseline withdrawal behaviors as a covariate, was conducted. The analysis revealed a significant main effect for drug condition [$F(1, 44)=7.66, p<0.01$], with the rats that had received nicotine displaying significantly more withdrawal behaviors than the rats that had received saline, and a main effect for sex [$F(1, 44)=12.78, p<0.01$] with females in both conditions displaying significantly more withdrawal behaviors than males in the corresponding conditions. There was no sex x drug condition interaction.

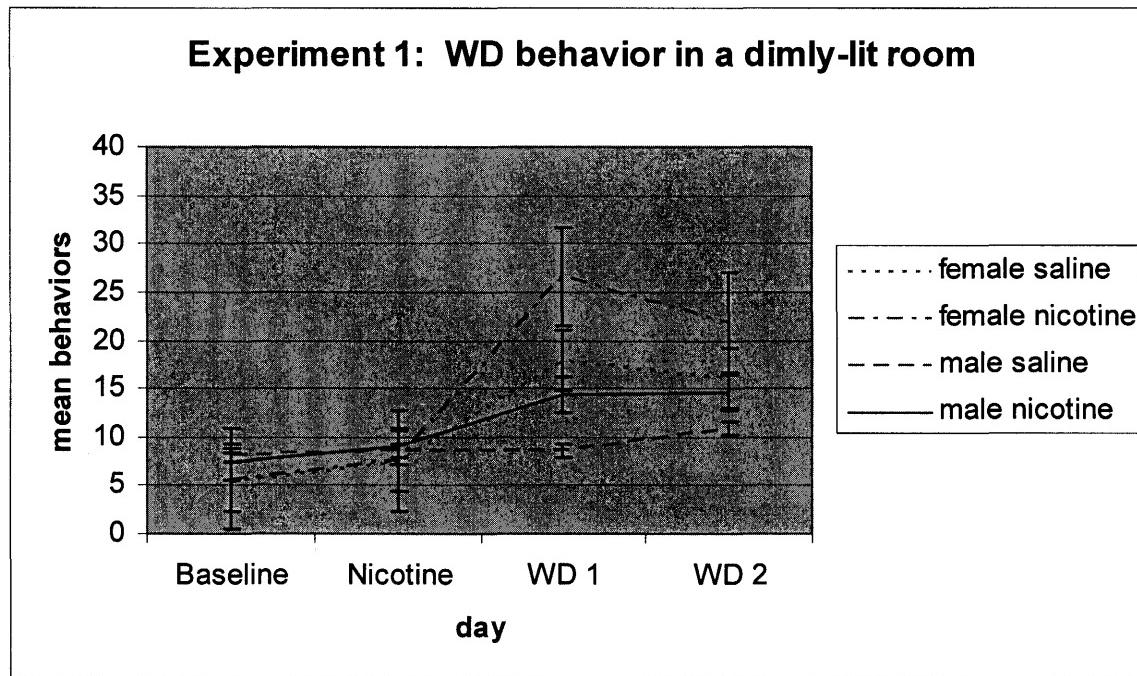


Figure 1

Experiment 1: Females

In addition, data for female and male rats were analyzed separately. A repeated-measures ANCOVA, using baseline withdrawal behaviors as a covariate, was conducted using time as the within-subject factor and drug condition as the between-subjects factor. This analysis revealed a main effect of drug condition [$F(1,21)=6.35, p<0.05$] for the entire experiment (both withdrawal days) with female rats that received nicotine displaying significantly more withdrawal behaviors than female rats that received saline. The statistical analyses are displayed in Appendix A.

Withdrawal Day One. For Withdrawal Day One, an ANCOVA using baseline as the covariate was conducted, with drug condition as the between-

subjects factor. The analysis revealed a main effect of drug condition [$F(1, 21)=8.94$, $p<0.01$], with female animals that received nicotine displaying more withdrawal behaviors than female animals that received saline.

Withdrawal Day Two. For Withdrawal Day Two, an ANCOVA using baseline as the covariate was conducted, with drug condition as the between-subjects factor. The analysis revealed that there was no main effect of drug condition for females on the second withdrawal day [$F(1, 21)= 3.81$, n.s.], with animals that received nicotine displaying an amount of withdrawal behaviors that did not differ significantly from animals that received saline.

Estrous. A mixed model approach was used to examine the contribution of estrous cycle in explaining both sources of variability in total withdrawal behaviors across four of the five observation days: Baseline, Nicotine, Withdrawal Day One, and Withdrawal Day Three. (Estrous measurements were not collected on Withdrawal Day Two). Cycle was not significantly associated with Withdrawal Behavior [$F(3, 90)=0.735$, n.s.].

Locomotor. An Analysis of Variance (ANOVA), using drug condition as the between-subjects factor, was conducted to determine whether there were significant differences in overall locomotor activity before administration of the drug. Female saline rats did not differ from the female nicotine rats on measures of horizontal activity [$F(1,22)=0.102$, n.s.], vertical activity [$F(1,22)=0.255$, n.s.], and center time [$F(1,22)=2.456$, n.s.].

Body weight. A Repeated-Measures Analysis of Variance, using phase as the within-subject factor and drug condition as the between-subjects factor, was

conducted to analyze body weight. The effect of drug condition x phase on females was not significant [$F(1,22)=1.132$, n.s.].

Experiment 1: Males

Data from the males were also analyzed separately from female data with a repeated-measures ANCOVA using baseline withdrawal behaviors as a covariate, using time as the within-subject factor and drug condition as the between-subjects factor. The analysis revealed a significant main effect of drug condition [$F(1, 21)=9.204$, $p<0.01$] for the entire experiment (including both withdrawal days) with male rats that received nicotine displaying significantly more withdrawal behaviors than male rats that received saline. The results for males are displayed in Figure 1.2. The statistical analyses are displayed in Appendix A.

Additionally, ANCOVAs using baseline withdrawal behaviors as the covariate were conducted for each of the two withdrawal days to determine how significant effects may have differed across withdrawal days.

Withdrawal Day One. For Withdrawal Day One, an ANCOVA using baseline as the covariate was conducted, with drug condition as the between-subjects factor. The analysis revealed a main effect of drug condition [$F(1,22)=5.96$, $p<0.05$], with male animals that received nicotine displaying more withdrawal behaviors than male animals that received saline.

Withdrawal Day Two. For Withdrawal Day Two, an ANCOVA using baseline as the covariate was conducted, using drug condition as the between-subjects factor. The analysis revealed a main effect of drug condition

[$F(1,22)=4.21$, $p=0.053$], with male animals that received nicotine displaying more withdrawal behaviors than male animals that received nicotine.

Locomotor. A Repeated-Measures Analysis of Variance, using phase as the within-subject factor and drug condition as the between-subjects factor, was conducted to determine whether there were significant differences in overall locomotor activity. Male saline rats did not differ from the male nicotine rats on measures of horizontal activity [$F(1,22)=0.733$, n.s.], vertical activity [$F(1,22)=0.590$, n.s.], and center time [$F(1,22)=3.020$, n.s.].

Body weight. A Repeated-Measures Analysis of Variance, using phase as the within-subject factor and drug condition as the between-subjects factor, was conducted to analyze body weight. The effect of drug condition x phase was not significant [$F(1,21)=2.429$, n.s.].

DISCUSSION

Withdrawal Observations

Experiment 1 was conducted in an environment that was similar to home cages and was designed to be non-stressful. Observations were conducted in a darkened room in cages with bedding, and the observation period was 15 minutes long. Rats that received nicotine displayed more withdrawal behaviors than rats that received saline. Additionally, females displayed more overall withdrawal behaviors than males, regardless of drug condition, but there was no sex x drug interaction. While nicotine rats displayed more overall somatic behavioral signs, the signs that differed most in their occurrence between saline and nicotine rats were eyeblinks, abnormal grooming, and abnormal posture.

In the animal studies of male nicotine withdrawal upon which the methodology of the present experiments is based, Malin et al. (1992, 1994, 2006) found a greater magnitude of withdrawal behavior in males than was found in males in the first experiment of the present series. In a personal communication (Malin, 2006), it was revealed that there were important differences between Malin's experiment (1992, 1994, 2006) and the present Experiment 1 in the environments in which withdrawal behaviors were observed. Male withdrawal behavior in Experiment 1 was of a smaller magnitude than male withdrawal behavior in Malin's experiments (1992, 1994, 2006). It was postulated that nicotine withdrawal may be affected by the environment in which withdrawal behaviors are observed. For this reason, nicotine withdrawal symptoms were

observed in the present Experiment 2 in an environment that closely modeled that used by Malin et al. (1992, 1994, 2006), as will be discussed presently.

Hypothesis 1 was confirmed because rats that received nicotine displayed more nicotine withdrawal symptoms than rats that received saline. Hypothesis 2 was partially confirmed because female rats that received nicotine had more withdrawal symptoms than male rats that received nicotine. Specifically, female rats had a greater magnitude of withdrawal behaviors than male rats on both withdrawal days, consistent with Hypothesis 2a. However, there was no sex x drug interaction in withdrawal behavior. Hypothesis 2B was not confirmed.

Body Weight

In Experiment 1, there was no significant effect of nicotine on body weight across phases, although there was a trend for the females that received nicotine to weigh less than the females that received saline.

Locomotor Activity

There were no differences in locomotor activity between the saline and nicotine groups in Experiment 1. As a result, it is unlikely that differences in withdrawal behaviors observed between the saline and nicotine conditions were due to changes in overall locomotor activity, but instead resulted from increases in the specific nicotine withdrawal behaviors.

Estrous

Cycle was not significantly associated with withdrawal behavior in Experiment 1.

All statistical analyses are presented in tables in Appendix A.

Experiment 2

Male and Female Rat Nicotine Withdrawal Observed in a Brightly-Lit Environment in Cages without Bedding

Overview

The purpose of this experiment was to examine male and female rats' nicotine withdrawal in an environment that differed from that of Experiment 1. Although it is not stated in any published reports (Malin, 1992, 1994, 2006), Malin observed rats for 20 minutes each in a larger rat polycarbonate shoebox cage without bedding in a brightly lit room (Dr. Malin, personal communication, 2006). This environment differed from the environment used in Experiment 1, in which rats were observed for 15 minutes at a time in a polycarbonate shoebox cage with bedding in a dimly-lit room. The bright lights in the room may have been a source of environmental stress for the rats. Because rats are nocturnal animals that naturally avoid the light, exposure to bright light is a stressor for rats, and has been used as a stressor in experimental investigations (e.g., Slawecki, 2005). Because stress results in increased nicotine administration, it is possible that environmental conditions can alter nicotine withdrawal.

The independent variables in the present experiment were sex (male, female) and drug condition (nicotine, saline), and the dependent variables were observed withdrawal behaviors, body weight, and locomotor activity. The present experiment was a 2 (sex) x 2 (drug condition) x 5 (time) mixed model, with sex and drug condition as between-subjects factors and time of observation as the within-subject factor.

The days on which each measure occurred are illustrated in the following timeline:

Day 1: rats arrive
Days 2-3: gentling
Day 4: estrous measurements begin (females)
Day 6: baseline locomotor activity
Day 7: baseline withdrawal observations
Day 8: pump implant
Day 14: nicotine withdrawal observations
Day 15: pump explant
Day 16: withdrawal day one observations
Day 17: withdrawal day two observations
Day 24: estrous measurements end (females)
Day 24: euthanasia

METHODS

Subjects

Subjects were 24 Sprague Dawley males and 24 Sprague Dawley females. All rats were approximately 65 days old. Upon arrival, the males mean weight was 286.77 grams, and the females' mean weight was 200.21 grams. Housing conditions in Experiment 2 were identical to the housing conditions in Experiment 1. Males and females were housed in the same room to regularize estrous cycling.

Baseline Phase

The baseline phase lasted for one week (7 days) after rats' arrival. In the first two days after arrival, rats were gentled and acclimated to the activity chamber. Daily estrous measurements commenced on the sixth day of the baseline phase. Rats were weighed every other day at the same time as estrous measurements, with the first day of body weight measurement on the third day after arrival. On the third day after arrival, rats were placed in the same individual activity chambers as were used in experiment 1 for one hour to record horizontal and vertical activity. Baseline observations were conducted on the sixth day of baseline.

Locomotor activity measurement

As in Experiment 1, rats' locomotor activity was measured to account for differences in overall general activity.

Estrous measurements and male handling

As in Experiment 1, estrous measurements were conducted on the female rats to determine if withdrawal behavior was related to estrous cycle phase. The measurements were taken on Mondays, Wednesdays, and Fridays at 9 a.m. for 18 days, beginning 6 days after the rats' arrival. A flushing technique was used to collect vaginal epithelial cells from each rat in the same procedure as in Experiment 1. The cells were also mounted and dyed on microscope slides and rated by independent raters in the same procedure as was used in Experiment 1.

To control for any effect of extra handling on the females, the male rats were held for approximately 5 seconds each in the same position as the females on the days of estrous measurements.

Observation Period

Observations were conducted from 1200 hours to 1600 hours on the last day of baseline phase, the last day of nicotine phase, and the first three days of the withdrawal phase (20 hours, 44 hours, and 68 hours after pump explant). In contrast to Experiment 1, the observations were conducted with rats in polycarbonate shoebox cages without bedding in a brightly lit room illuminated by overhead fluorescent lights, with each observation period lasting 20 minutes.

Animals were observed in clean polycarbonate shoebox cages that were larger than those used in experiment 1 (46 cm x 36 cm x 20 cm) in a separate room from the housing room. Consistent with experiment 1, withdrawal behaviors from Malin et al.'s (1992) model were quantified during observations. These behaviors included abnormal posture, abnormal grooming, "wet dog

shakes," ptosis, mouthing/teeth chattering, and diarrhea. Other behaviors that appeared abnormal were counted as "other." A detailed description of behaviors, which was distributed to raters on each observation day, is included in Appendix A.

Drug Administration Phase

Alzet osmotic pumps (Model 2ML2, Alza Corp., Palo Alto, CA) filled with 3.16 mg/kg/day nicotine bitartrate solution or 0.9% NaCl (physiological saline) were used to administer nicotine or saline continuously for seven days at 5 microliters/hour.

Pumps were implanted seven days after the rats' arrival using an identical procedure to that used in Experiment 1. The drug administration phase began after the pump implant. During this phase, which lasted one week, rats received a continuous flow of nicotine bitartrate or physiological saline (0.9% NaCl) at 5.25 microliters/hour for a dosage of 3.16 mg/kg/day (expressed as nicotine base). Estrous measurements and nicotine measurements continued to be taken at their usual times. Observations of withdrawal behavior during drug administration (nicotine or saline) took place on the sixth day of the drug administration phase in the manner described above. The drug administration phase ended with the pump explant, which took place from 1800 hours to 2200 hours on the last day of the nicotine phase, one week after pump implant.

Two locomotor activity measurements were taken during drug administration phase in Experiment 2. On the first and third days of the drug

administration phase, rats were placed in the individual activity chambers for one hour to record horizontal and vertical activity.

Withdrawal Phase

Pumps were explanted seven days after they were implanted using an identical procedure to that which was used in experiment 1. Withdrawal phase began immediately after pump explant. Rats were observed 20 hours after pump removal, in the middle of the optimal 18 to 22 hour window for observing withdrawal behaviors described by Malin, et al. (1992).

Observations

Three withdrawal phase observations were conducted in a manner identical to the observations in the baseline and administration phases. In the first observation, rats were observed approximately 20 hours post pump removal. The second withdrawal observation took place 24 hours after the first withdrawal observation. The third withdrawal observation took place 24 hours after the second withdrawal observation.

Locomotor Activity

Locomotor activity was collected in a manner that was identical to locomotor activity collection in the baseline and administration phases.

Estrous and body weight measurements

Estrous and body weight measurements were carried out until two weeks after the second withdrawal day.

Materials and Equipment

Activity Chambers

Animals were placed in individual electronic physical activity monitoring chambers of the Omnitech Electronics Digiscan infrared photocell system [Test box model RXYZCM (16 TAO); Omnitech Electronics, Columbus, OH] for one hour to measure open field locomotor activity and record vertical and horizontal movement via a grid of infrared light beams. Equally spaced beams traversed the plastic arenas (40 x 40 x 30 cm) from front to back and left to right. Fifteen pairs of infrared photocells are located every 2.5 cm from left to right and from front to back in a plane 2 cm above the floor o the chamber to measure horizontal movement. An additional 15 pairs of infrared photocells are located every 2.5 cm from left to right 10.5 cm above the floor of the chamber to measure vertical movement. Dependent variables included center time, horizontal activity, and vertical activity. The body of the animal placed in the chambers broke the beams, revealing any horizontal and vertical movement and center time. These parameters were automatically calculated based on beam breaks in twelve five minute bins, and automatically transferred to a personal computer via an Omnitech analyzer (Model DCM-8-BBU). Analyses were performed on total scores for each dependent variable which were calculated by summing the scores for each parameter recorded every five minutes. The apparatus monitored animal activity continuously for a total testing period of 1 hour, collecting data in 5 minute bins. Several activity-related variables were examined including total horizontal activity, total vertical activity, and center time.

Estrous measurements

The materials for the estrous measurements included a slide tray, flamed disposable 5 ¾" Fisher brand pasteur pipets, 2 ml Sigma-Aldrich brand rubber bulbs, Fisher brand frosted microscope slides divided into eight sections each with a diamond-tipped pen, and 0.9% NaCl (physiological saline).

Estrous slide staining

The materials used for estrous slide staining included the chemicals 100% Methanol, Hematoxylin, Eosin Y, Permount, Xylene, and 100% Alcohol (200 proof). The non-chemical materials used included disposable pipettes, rubber bulbs, blunt forceps, a slide tray, a funnel, and Coplin jars.

Nicotine or Saline administration

Alzet osmotic pumps (Model 2ML2) filled with 3.16 mg/kg/day nicotine bitartrate solution or 0.9% NaCl (physiological saline) were used to administer nicotine or saline continuously for seven days at approximately five microliters/hour. The nicotine dosage, 3.16 mg/kg/day, was used because that dosage was determined by Malin et al. (1992, 1994, 2001), Phillips et al. (2004), and O'Dell et al. (2004, 2006) to be effective for eliciting withdrawal behaviors after cessation of continuous administration.

RESULTS

A repeated-measures ANCOVA, using baseline as the covariate, was conducted on the three cessation days using drug condition and sex as the between-subjects factors and time as the within-subject factor. The analysis revealed a main effect of drug condition [$F(1, 44)=18.73, p<0.01$], with animals that received nicotine displaying significantly more withdrawal behaviors than animals that received saline, but there was no significant effect of sex [$F(1, 44)=2.81, \text{n.s.}$].

Additionally, ANCOVAs using baseline withdrawal behaviors as a covariate were conducted for each of the three withdrawal days to determine how significant effects may have differed across withdrawal days. The withdrawal behavior results of Experiment 2 are displayed in Figure 2. All tests were two-tailed. Alpha levels were set at 0.05.

Withdrawal Day One. For the separate analysis of Withdrawal Day One, an ANCOVA, using baseline as the covariate, was conducted using time as the within-subject factor and drug condition and sex as the between-subjects factors. The analysis revealed a main effect of drug condition [$F(1, 44)=29.63, p<0.01$], with rats that received nicotine displaying more withdrawal behaviors than rats that received saline, and no effect of sex [$F(1, 44)=1.361, \text{n.s.}$], with males and females displaying similar amounts of withdrawal behaviors.

Withdrawal Day Two. For the separate analysis of Withdrawal Day Two, a repeated-measures ANCOVA, using baseline as the covariate, was conducted using time as the within-subject factor and drug condition and sex as the between-subjects factors. The analysis revealed a main effect of drug condition [$F(1, 44)=7.51, p<0.01$], with rats that received nicotine displaying more withdrawal behaviors than rats that received saline, and no effect of sex [$F(1, 44)=0.46, \text{n.s.}$], with males and females displaying similar amounts of withdrawal behaviors.

Withdrawal Day Three. For the separate analysis of Withdrawal Day Three, a repeated-measures ANCOVA, using baseline as the covariate, was conducted using time as the within-subject factor and drug condition and sex as the between-subjects factors. The analysis revealed a main effect of sex [$F(1, 44)=8.07, p<0.01$], with male rats from both drug conditions displaying more withdrawal behaviors than female rats from the corresponding drug conditions, and no effect of drug condition [$F(1, 44)=3.19, \text{n.s.}$], with rats that received saline and rats that received nicotine displaying withdrawal behaviors that were not significantly different in amount.

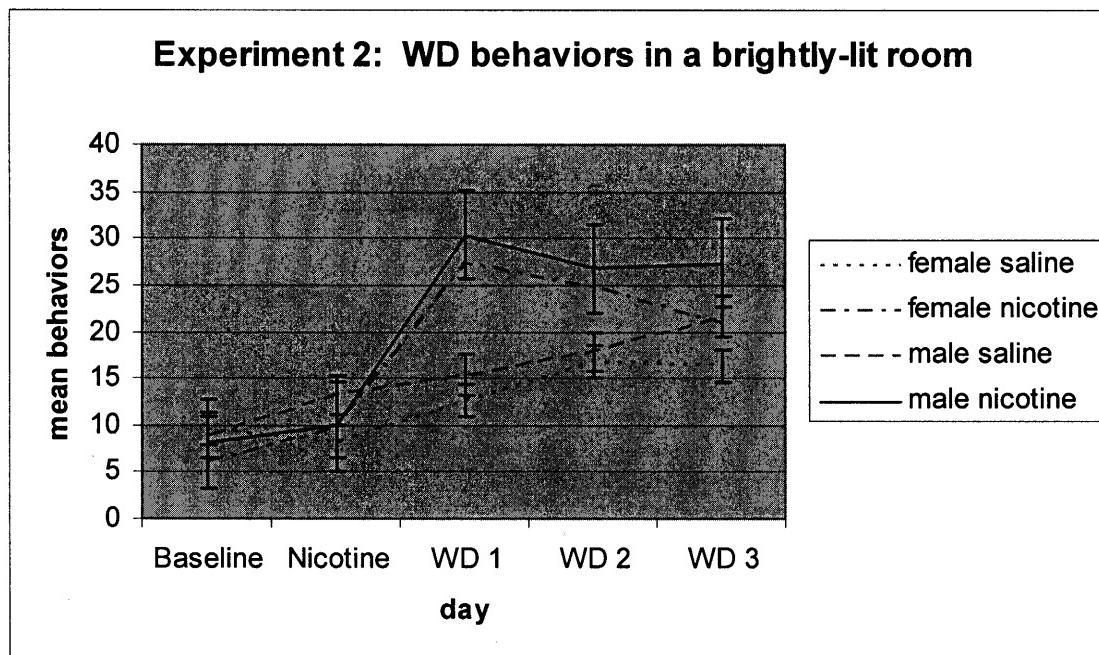


Figure 2

Estrous analysis. A mixed model approach was used to explain between and within-subject variability and the contribution of estrous cycle in explaining both sources of variability in total withdrawal behaviors across four of the five observation days: Baseline, Nicotine, Withdrawal Day One, and Withdrawal Day Three. (Estrous measurements were not collected on Withdrawal Day Two). Cycle was not significantly associated with Withdrawal Behavior [$F(3, 90)=2.110$, n.s.]. The addition of cycle to the mixed model yielded a 2.8% reduction in total variance, with a 2.6% reduction in within-subjects variance and a 3.6% reduction in between-subjects variance.

Body weight. A Repeated-Measures Analysis of Variance, using phase as the within-subject factor and drug condition as the between-subjects factor, was

conducted to analyze male and female body weight separately. There was a significant effect of drug condition x phase for both females [$F(1,22)=5.921$, $p<0.01$] and males [$F(1,22)=3.746$, $p<0.05$].

Locomotor. A Repeated-Measures Analysis of Variance, using phase as the within-subject factor and drug condition as the between-subjects factor, was conducted to determine whether there were significant differences in overall locomotor activity. Saline rats did not differ from the nicotine rats on measures of horizontal activity [$F(1,46)=3.342$, n.s.], vertical activity [$F(1,46)=0.013$, n.s.], and center time [$F(1,46)=3.856$, n.s.].

DISCUSSION

In Experiment 2, withdrawal behavior observations were conducted in a brightly-lit room. Rats were observed for 20 minutes at a time in large cages with no bedding. It is possible that this atmosphere provided a mild stressor (Slawecki et al., 2005), which seems to have exacerbated the effects of withdrawal in the male rats, but not the female rats. In Experiment 1, mean withdrawal behavior of males was smaller than mean withdrawal behavior of females, but male and female rats had similar mean withdrawal behavior in Experiment 2. However, as later discussed, when adjusting for differences between the two experiments in the amount of time rats were observed, it is revealed that the effect of the environment was greatest in female rat withdrawal behavior.

Withdrawal Observations

Animals that received nicotine displayed significantly more withdrawal behaviors than animals that received saline. There was no effect of sex in Experiment 2; males and females displayed a similar amount of withdrawal behaviors. In Experiment 2, observations were conducted in a brightly-lit room that was illuminated by an overhead fluorescent light. Subjects were observed in large polycarbonate cages without bedding. There was no significant effect of sex or sex x drug interaction. While nicotine rats displayed more overall somatic behavioral signs, the signs that differed most in their occurrence in Experiment 2

between saline and nicotine rats were eyeblinks, abnormal grooming, and whole body shakes.

Body weight

In Experiment 2, there was a significant effect of nicotine on body weight across drug phases, with animals that received nicotine weighing less than animals that received saline. Females that received nicotine weighed significantly less than females that received saline during the nicotine phase, but there was only a non-significant trend for the males during nicotine phase.

Locomotor Activity

There were no differences in locomotor activity between the saline and nicotine groups in Experiment 2. As a result, it is unlikely that differences in withdrawal behaviors observed between the saline and nicotine conditions were due to changes in overall locomotor activity, but instead resulted from increases in the specific nicotine withdrawal behaviors identified by Malin et al. (1992).

Estrous

Cycle was not significantly associated with withdrawal behavior in Experiment 1 or Experiment 2. This means that the effect of estrous cycle on withdrawal behavior in female rats was minimal. In fact, the addition of cycle to the mixed model yielded only a 2.8% reduction in total variance.

Experiments 1 and 2

The purpose of the present experiments was to examine nicotine withdrawal in male and female adult rats in different environments. Animals that received nicotine displayed more withdrawal behaviors than animals that received saline. There were sex differences in the dimly-lit environment (Experiment 1), such that females of both drug conditions displayed more withdrawal behaviors than males.

CONFIRMATION OF HYPOTHESES

Hypothesis 1. The hypothesis that rats that received nicotine would display more withdrawal than rats that received saline after pump removal was confirmed. Both male and female rats that received nicotine reliably displayed more withdrawal behaviors than their male and female counterparts that received saline.

Hypothesis 2. The hypothesis that female rats would display more withdrawal behavior overall than males was partially confirmed. Female rats displayed more withdrawal behavior than males in a dimly-lit environment with cage bedding, but there was no sex difference in amount of withdrawal behavior in a brightly-lit environment with no cage bedding as the magnitude of males withdrawal behavior increased in Experiment 2, while the magnitude of the female withdrawal behavior was the same in both experiments.

Hypothesis 2A. The hypothesis that female rats would display a greater magnitude of withdrawal behavior than males was confirmed in Experiment 1, in which observations were conducted in a dimly-lit environment, but was not confirmed in Experiment 2, in which observations were conducted in a brightly-lit environment. However, there was no sex x drug interaction.

Hypothesis 2B. The hypothesis that female rats would have a longer duration of withdrawal was not confirmed in either experiment.

Hypothesis 3. The hypothesis that the effect of withdrawal would last longer in females than males was not confirmed.

Hypothesis 4. It was hypothesized nicotine administration would decrease body weight in males and females. The hypothesis that nicotine administration would decrease body weight was confirmed for females with a non-significant trend, but not males, in Experiment 1. The hypothesis that nicotine would decrease body weight was confirmed for both males and females in Experiment 2.

Comparison of Results: Experiments 1 and 2

Mean withdrawal behaviors are displayed in Table 28 in Appendix A. To control for the slightly longer observation period used in Experiment 2, ratios were calculated for each observation period in which withdrawal behaviors displayed by the nicotine group were divided by withdrawal behaviors displayed by the saline group during that period. The ratios of withdrawal behaviors for males and females are listed in Table 1.

After adjusting for the longer observation period in Experiment 2, it is clear that withdrawal behaviors were greater in magnitude in the brightly-lit environment in both males and females, but the effect of the environment was greatest in the female rats. In male rats, withdrawal behaviors were increased in the brightly-lit environment from the dimly-lit environment by 31%, but were increased in female rats by 67%. Comparison of these ratios suggests that the environment had a greater effect on withdrawal behavior in female rats.

Table 29. Nicotine/Saline Withdrawal Ratios

	Experiment 1 Dimly-lit environment	Experiment 2 Brightly-lit environment	WD1	WD2	WD3
males	1.67	1.34	1.98	1.51	1.27
females	1.49	1.36	2.16	1.49	1.26

Experiments 1 and 2: Nicotine/Saline Withdrawal Behavior Ratios

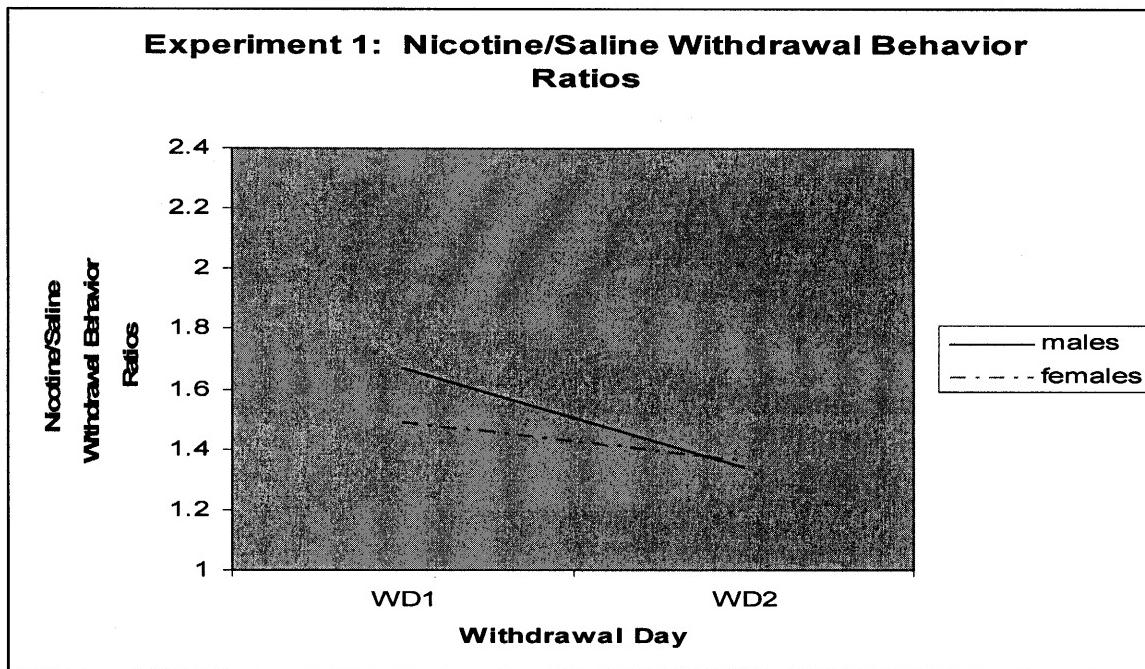


Figure 3

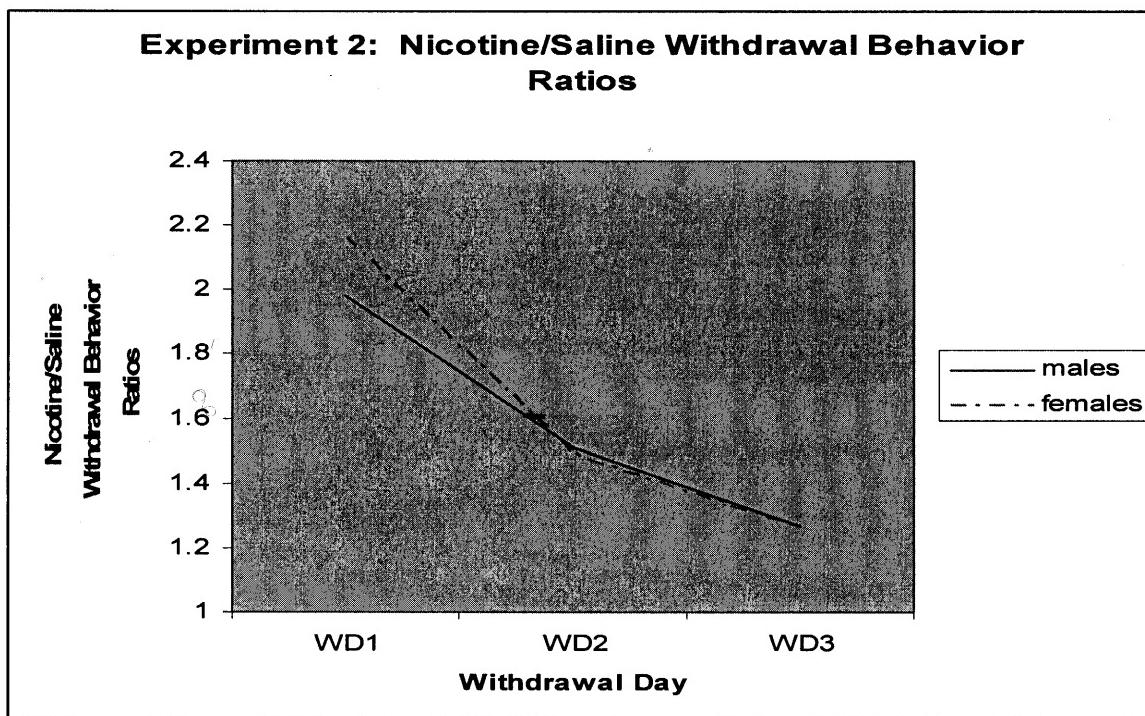


Figure 4

GENERAL DISCUSSION

In Experiment 1 there were sex differences in nicotine withdrawal with females having greater mean withdrawal symptoms than males. In Experiment 2, when the rats were observed in a brightly-lit environment, the magnitude of males' withdrawal behavior was increased whereas the magnitude of female's withdrawal behavior was similar to that displayed in the home-like environment. The observation period in Experiment 2 was 5 minutes longer than the observation period in Experiment 1, which could have contributed to differences in the amount of withdrawal behavior observed. However, this difference was accounted for by the calculation of withdrawal behavior ratios for each observation period, in which withdrawal behaviors displayed by the nicotine group were divided by withdrawal behaviors displayed by the saline group during that period. These calculations revealed that the brightly-lit environment had a greater effect in increasing female rat withdrawal behavior than male rat withdrawal behavior. There was a main effect of drug condition in both experiments, but the main effect of sex only occurred in Experiment 1. However, there was no sex x drug interaction in either experiment.

It is likely that the differences in patterns of withdrawal behavior in Experiments One and Two resulted from differences in the environments in which observations were conducted. In Experiment 1, the animals were observed in a dimly-lit room in standard sized cages with bedding. The observation environment was constructed to model the home cage as closely as possible, with the intention of maximizing the rats' comfort. This environment

contrasted sharply with the observation environment in the second experiment, in which rats were observed in a brightly-lit room in larger cages without bedding. Because bright lights can be stressful to rats (Slawecki, 2005; Frye and Orecki, 2002), it is likely that the environment in Experiment 2 provided a mild stressor for the rats.

Interestingly, in the brightly-lit environment (Experiment 2) there was a main effect of drug condition, with rats that received nicotine demonstrating significantly more withdrawal behavior than rats that received saline, although there was no significant effect of sex overall.

Use of animal model

Human studies involve self-report, expectation, and recall bias, and are limited by ethical considerations. For instance, a true experiment to study sex differences in nicotine withdrawal symptoms would require the randomization of cigarette smoking/addiction or saline administration to drug naive humans, followed by a cessation of cigarette smoking or saline administration to examine nicotine withdrawal symptoms. However, randomizing humans who are not already addicted to a nicotine addiction condition clearly raises ethical concerns.

Studies comparing the effects of the environment and stress on nicotine withdrawal in men and women must be conducted to ensure that the present results generalize to humans. If the results of the present experiments generalize to humans, then these results would have important implications for advising men and women who want to quit smoking.

Limitations

One limitation of the present study is a five-minute difference in the amount of observation time from Experiment 1 to Experiment 2. Rats in Experiment 1 were observed for fifteen minutes at a time, while rats in Experiment 2 were observed for twenty minutes. However, this difference was accounted for by the calculation of withdrawal behavior ratios for each observation period, in which withdrawal behaviors displayed by the nicotine group were divided by withdrawal behaviors displayed by the saline group during that period. While this difference in time may have contributed to the increase in the amount of withdrawal behaviors in Experiment 2, it is unlikely that the time difference is the sole reason for the increase in withdrawal behaviors because the increase in the amount of withdrawal behaviors was only seen in males, while the amount of withdrawal behaviors displayed by females in Experiment 2 is remarkably similar to that which was displayed in Experiment 1. If time were the factor contributing to the increase in withdrawal behaviors, then one would expect to see the difference reflected in both males and females.

A second limitation of the present experiment is that while it was speculated that environmental stress affected withdrawal behavior, stress was not manipulated or assessed biologically or behaviorally.

A third limitation of the present study is that locomotor data were not collected for the females on Withdrawal Day One of Experiment 1, and locomotor data were lost for a few of the subjects on Withdrawal Day One of Experiment 2.

Locomotor data was collected to account for effects on nicotine withdrawal on overall activity, in order to determine whether differences in nicotine withdrawal behavior were merely a reflection of increased overall activity rather than an increase in specific nicotine withdrawal behaviors (Malin et al., 1992). While locomotor data were not collected for the females in Experiment 1, it was collected for females in Experiment 2, where it was revealed that group differences in withdrawal behaviors were not a reflection of overall differences in locomotor activity.

Summary and Implications

Cigarette smoking is a major public health concern, with approximately one-fifth of U.S. adults smoking cigarettes (CDC, 2006). Cigarette smoking prevalence is similar between men and women, but women are less successful than men at quitting smoking (e.g. Perkins, 2001). The present experiment was conducted to determine whether reported sex differences in withdrawal (e.g. Shiffman, 1979), which are relevant to tobacco use and treatment, may be based on biological sex differences per se or environmental influences, using rats to control for purely psychological phenomena.

There were three major findings in the present experiment. First, nicotine withdrawal exists, and can be modeled in rodents, consistent with the work of Malin et al. (1992, 1994, 2006), Phillips et al. (2004), and O'Dell et al. (2004, 2006). Second, nicotine withdrawal exists in males and females, although it is somewhat different in different environments. The present experiment was the

first to compare males and females in an animal model of nicotine withdrawal. Third, environment modulates the expression of nicotine withdrawal behaviors differently in males and females. Both males and females displayed more withdrawal behaviors in a brightly lit environment than in a dimly-lit environment, but the effect of the environment on withdrawal behavior was greater in female rats.

The finding that nicotine withdrawal exists and can be modeled in rodents is consistent with the work of Malin et al. (1992, 1994, 2006), Phillips et al. (2004), and O'Dell et al. (2004, 2006) who found nicotine withdrawal in male adult rats and compared withdrawal in adult and adolescent rats, respectively. The second finding, that nicotine withdrawal exists in both males and females in a rodent model, is a novel finding. The finding that the environment affects nicotine withdrawal, especially in females, is also a novel finding.

In summary, adult male and female rats experienced nicotine withdrawal after cessation of continuously administered nicotine, indicating that both males and females experienced nicotine dependence. Body weight was decreased by nicotine in both males and females, consistent with Faraday et al. (2001), although these results were only statistically significant in females, consistent with the research of Grunberg et al., (1984, 1986). The body weight results confirm that the rats received nicotine. Differences between the saline and nicotine groups in withdrawal behaviors did not result from differences in overall locomotor activity, and female withdrawal behavior was not significantly affected by estrous cycle phase.

There was a main effect of sex in the dimly-lit environment of Experiment 1 that disappeared in the brightly-lit environment of Experiment 2. Males displayed a greater magnitude of withdrawal behaviors in the brightly-lit environment than the dimly-lit environment, while females displayed a similar magnitude of withdrawal behaviors in both environments. However, when the longer observation period in Experiment 2 was accounted for by the creation of withdrawal ratios, a greater effect of the environment on withdrawal behaviors in females was revealed. This finding is consistent with the work of Perkins (1999), who reported that environmental context plays a greater role in determining the perception of nicotine effects in women than men. If the environmental context is important for perception of nicotine effects in women, it follows that environmental context may also affect nicotine withdrawal in women. The results of the present experiment, in which the environment had a greater effect on withdrawal behavior in female rats, supports this suggestion.

A main element of the environment in Experiment 2, bright lights, is commonly used in research as a stressor for rats (Slawecki, 2005; Frye and Orecki, 2002). Al' Absi (2006) reports that intensity of withdrawal symptoms after exposure to acute stress is a consistent predictor of smoking relapse in women. While stress was not assessed biologically in the present experiments, it is likely that the observation environment in Experiment 2 created a stressor that exacerbated withdrawal. Therefore, the level of physiological addiction, as measured by withdrawal behaviors, was the same in both males and females, although potential environmental stressors may exacerbate withdrawal

symptomatology, particularly in females. If these results extrapolate to humans, then women may be less successful at smoking cessation because their withdrawal symptomatology is increased by stress encountered in daily life. In addition, if the results extrapolate to humans, then they suggest that women may be more sensitive than men to environmental stress, especially during nicotine cessation. However, these assertions are speculative in nature, as no biological measures of stress were included in the present experiment.

Future directions

Future series of experiments of nicotine withdrawal differences in rats should be conducted using the same observation period time for all experiments in the series to make comparisons more direct. In the present experiment, it was speculated that nicotine withdrawal may have been affected by environmental stress. However, stress was not assessed biologically or behaviorally in the present experiment. In future studies, the effects of environmental manipulations on corticosteroid levels should be assessed to determine whether the environment was stressful. It is clear that cigarette smokers smoke more under stress (Schachter et al., 1977; USSGR, 1988; Grunberg & Baum, 1985; Pomerleau and Pomerleau, 1987; Kassel, Stroud, & Patronis, 2003). However, the mechanism by which stress increases smoking behavior is unknown. Future research should manipulate stress harshly as an independent variable and determine the effect of stress on nicotine withdrawal in male and female rats. The effect of the environment and stress on nicotine withdrawal in humans is an

important area in which to conduct future research, to determine whether the results of the present experimental series generalize to humans. In addition, the effect of stress on another action of nicotine, behavioral sensitization, should be examined to determine if stress affects the rewarding properties of nicotine. Impulsivity is a psychological and behavioral construct that is implicated in substance abuse, as well as a variety of other detrimental behaviors. Another interesting future direction would be to examine the effect of stress on nicotine withdrawal and actions of nicotine in an animal model of impulsivity. Future research should be aimed at elucidating the relation between sex hormone cycling and nicotine withdrawal in females by manipulating sex hormone levels and examining the effects of these manipulations on nicotine withdrawal. Lastly, future research should be aimed at using animal models to experimentally test other factors predicting smoking cessation outcome, such as social environment (e.g., Lee and Kahende, 2007).

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APPENDIX A (Withdrawal Behavior Criteria)

Appendix A - CRITERIA FOR ADOLESCENT NICOTINE WITHDRAWAL SYMPTOMS

1. **Wet-Dog Shakes/Tremors:** animal vigorously shakes the head or the entire body (wet-dog) shake; similar to the behavior of a wet dog shaking to remove excess water. Animal “shivers” in entire body, or specific body parts, particular the cheek (tremors). Each episode is counted as one occurrence or symptom.
2. **Diarrhea:** Loose or runny stool. Each episode is counted as once occurrence or symptom.
3. **Mouth and Teeth Chattering:** Rapid chattering of the teeth or empty-mouth chewing movements. Each episode is counted as one occurrence or symptom.
4. **Ptosis:** Slackening or relaxing of the facial muscles; especially apparent as a heavy-lidded or “sleepy” appearance. Each episode is counted as one occurrence or symptom, but occurrences are limited to a maximum of one per minute.
5. **Abnormal Grooming:** especially persistent or rough grooming behavior which may include chewing of the forepaws or other body parts, “violent” washing of the face and body, etc. Each episode is defined as counting to 10 seconds (e.g., 1-1000, 2-1000, 3-1000, etc.). After ten seconds, another episode begins (e.g. 30 seconds = 3 episodes).
6. **Abnormal Posture/Movement:** unusual postures or movements that may include writhing, twisting of the body while sitting or moving, etc. Each episode is counted as an occurrence or symptom.
7. **Other behaviors:** Miscellaneous abnormal behaviors which may include excessive yawning, seminal ejaculation, hind foot scratches, licking of the genitals, etc. Each episode is counted as one occurrence or symptoms. Please note on the symptoms checklist a description of each of these behaviors.

On a scale of 1 (low) – 5 (high) please indicate the following:

1. **Activity (Horizontal):** the animal’s activity around the cage (back and forth). A score of 1 is considered still for the whole time and 5 is considered to be racing around the cage for the whole observation period.
2. **Activity (Vertical):** the animal’s activity in the cage (up and down). A score of 1 is considered rarely being on his hind legs and a score of 5 is considered to be on his hind legs for a majority of the observation period.
3. **Health:** the animal’s health as defined by coloring (of the skin, fur and eyes), amount of activity, reaction to noises, gait, etc.

APPENDIX B (Tables)

APPENDIX B (Tables)

Experiment 1: males and females observed in low light with cage bedding

Withdrawal Observations

Table 1. Experiment 1, Withdrawal Observations, Baseline (BL): Analysis of Variance (ANOVA)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	0.422	1	0.422	0.051	.823
Sex	61.880	1	61.880	7.438	.009
Drug x Sex	2.755	1	2.755	0.331	.568
Error	366.062	44	8.320		

Table 2. Experiment 1, Withdrawal Observations, Drug or Saline Administration: ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	0.907	1	0.907	0.072	.789
Sex	17.763	1	17.763	1.417	.240
Drug x Sex	.403	1	.403	.032	.858
Error	551.645	44	12.537		

Table 3. Experiment 1, Withdrawal Observations: Repeated-measuresANCOVA with Baseline (BL) as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	866.369	1	866.369	16.39	.000
Sex	1571.895	1	1571.895	29.73	.000
Drug x Sex	35.733	1	35.733	0.676	0.416
Error	2273.457	43	52.871		

**Note: .000 indicates p < .0001

**Table 4. Experiment 1, Withdrawal Day One, Withdrawal Observations:
ANCOVA with BL as covariate**

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	626.596	1	626.596	15.31	.000
Sex	1199.894	1	1199.894	29.33	.000
Drug x Sex	25.961	1	25.961	0.634	0.43
Error	1759.462	43	40.918		

**Table 5. Experiment 1, Withdrawal Day Two, Withdrawal Observations:
ANCOVA with BL as covariate**

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	275.37	1	275.37	7.66	0.008
Sex	459.246	1	459.246	12.78	0.001
Drug x Sex	11.28	1	11.28	0.314	0.578
Error	1545.648	43	35.945		

**Note: .000 indicates $p < .0001$

Experiment 1: Females

Withdrawal Observations

Table 6. Experiment 1, Females: Withdrawal Observations, Repeated-measuresANCOVA with BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	171.051	1	171.051	6.351	.020
Error	565.575	21	26.932		

Table 7. Experiment 1, Females: Withdrawal Observations, Withdrawal Day One, ANCOVA using BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	453.856	1	453.856	8.94	.007
Error	1066.505	21	50.786		

Table 8. Experiment 1, Females: Withdrawal Observations, Withdrawal Day Two, ANCOVA using BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	200.202	1	200.202	3.81	.065
Error	1104.728	21	52.606		

Estrous

Table 9. Experiment 1, Estrous: Mixed Model: Type III Tests of Fixed Effects

Source	Numerator df	Denominator df	F	Sig.
Cycle	3	81.903	0.735	0.534

Experiment 1: Locomotor Activity Females

Table 10. Experiment 1, Female Locomotor Activity, Horizontal Activity: ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	2829753.375	1	2829753.375	0.102	.752
Error	608854813.583	22	27675218.799		

Table 11. Experiment 1, Female Locomotor Activity, Vertical Activity: ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	129948.167	1	129948.167	.255	.618
Error	608854813.583	22	27675218.799		

Table 12. Experiment 1, Female Locomotor Activity, Center Time: ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	266598.760	1	266598.760	2.456	.131
Error	2388351.709	22	108561.441		

**Body Weight
Females****Table 13. Experiment 1, Female Body Weight: Repeated-measuresANOVA**

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
drug x phase	38.485	2	19.243	1.132	0.332
Error (phase)	747.799	44	16.995		

Experiment 1: Males

Withdrawal Observations

Table 14. Experiment 1, Males: Withdrawal Observations, Repeated-measuresANCOVA with BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	273.845	1	273.845	9.204	0.006
Error	624.778	21	29.751		

Table 15. Experiment 1, Males: Withdrawal Day One, ANCOVA using BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	196.576	1	196.576	5.958	0.024
Error	692.898	21	32.995		

Table 16. Experiment 1, Males: Withdrawal Day Two, ANCOVA using BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	88.026	1	88.026	4.205	.053
Error	439.642	21	20.935		

**Experiment 1: Locomotor Activity
Males**

Table 17. Experiment 1, Male Locomotor Activity, Horizontal Activity: ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	88213230.021	1	88213230.021	3.716	.067
Error	522229392.458	22	23737699.657		

Table 18. Experiment 1, Male Locomotor Activity, Vertical Activity: ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	3876601.687	1	3876601.687	3.885	.061
Error	21953152.125	22	997870.551		

Table 19. Experiment 1, Male Locomotor Activity, Center Time: ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	748325.935	1	748325.935	3.020	.096
Error	299200819.667	22	13600037.258		

**Body Weight
Males**

Table 20. Experiment 1, Body Weight, Males: Repeated-measuresANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
drug condition	793.681	1	793.681	2.429	0.134
error	6862.681	21	326.794		

**Experiment 2: males and females observed in bright light
without cage bedding**

Table 19. Experiment 2, Withdrawal Observations: Repeated-measuresANCOVA with BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	2574.897	1	2574.897	18.733	.000
Sex	385.748	1	385.748	2.806	.101
error	5772.975	42	137.452		

Table 20. Experiment 2, Withdrawal Day One: Repeated-measuresANCOVA with BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	2294.208	1	2294.208	.415	.523
Sex	105.437	1	105.437	1.361	.250
error	3252.582	42	77.442		

Table 21. Experiment 2, Withdrawal Day Two: Repeated-measuresANCOVA with BL as covariate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	669.290	1	669.290	7.510	.009
Sex	40.558	1	40.558	.455	.504
error	3832.233	42	89.122		

Experiment 2: Locomotor Activity

Table 22. Experiment 2: Locomotor Activity, Horizontal Activity, ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
drug_condition	92267012.760	1	92267012.760	3.385	.072
Error	1253825737.396	46	27257081.248		

Table 23. Experiment 2: Locomotor Activity, Vertical Activity, ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	3876601.687	1	3876601.687	3.885	.061
Error	28838744.958	46	626929.238		

Table 24. Experiment 2: Locomotor Activity, Center time, ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
drug_condition	566775.075	1	566775.075	3.856	.057
Error	609423552.458	46	13248338.097		

Table 25. Experiment 2: Estrous analysis, Mixed Model, Type III Tests of Fixed Effects

Source	Numerator df	Denominator df	F	Sig.
Cycle	3	89.8233	2.110	0.104

Experiment 2

Table 26. Experiment 2: Body Weight, Males, Repeated-measuresANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	2123.912	1	2123.912	0.581	0.454
Error	80491.425	22	3658.701		

Table 27. Experiment 2: Body Weight, Females, Repeated-measuresANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug Condition	1617.373	1	1617.373	10.107	0.005
Error	3040.520	19	160.027		

Table 28. Mean withdrawal behaviors

Experiment 1: dimly lit environment				Experiment 2: brightly-lit environment							
	WD1	WD2	WD1		WD2	WD1	WD2		WD3	WD3	
	saline	nicotine	saline	nicotine	saline	nicotine	saline	nicotine	saline	nicotine	
males	8.5	14.3	10.9	14.6	15.3	30.3	17.8	26.8	21.6	27.3	
females	17.9	26.6	16	21.8	12.6	27.3	16.7	24.8	16.4	20.7	

Table 29. Nicotine/Saline Withdrawal Ratios

Experiment 1 brightly-lit environment		Experiment 2 dimly-lit environment		
		WD1	WD2	WD3
males		1.67	1.34	1.98
females		1.49	1.36	2.16
			1.51	1.27
			1.49	1.26